# VIRAL VECTORS

## *Gene Therapy and Neuroscience Applications*

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EXHIBIT A

CHAPTER 11

# *Adenoviral-Mediated Gene Transfer: Potential Therapeutic Applications*

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Effective methods for gene transfer to the central nervous system (CNS) will find applications in the treatment of neurologic deficits characteristic of many inborn errors of metabolism, as well as the ablation or growth inhibition of CNS neoplasms. This chapter provides a summary of a portion of the ongoing work in our laboratory to determine the capacity of recombinant adenovirus to correct enzyme defects *in vivo*, and to ameliorate existing and/or ongoing neuropathology in animal models of disease.

## I. Application to Inborn Errors with Neurologic Involvement

Many inborn errors have as a sequelae of the disorder, a severe and often devastating neurologic component. Representative of such disorders are deficiencies of purine metabolism and the mucopolysaccharidoses (MPS), or more specifically, the Lesch–Nyhan syndrome (LNS; Lesch and Nyhan, 1964) and β-glucuronidase deficiency (MPS VII; Sly syndrome; Sly *et al.*, 1973), respectively. Both disorders result in a very poor quality of life and shortened life span, resulting from numerous movement and intellectual deficits that often require specialized care.

Therapies directed toward correction of the MPS or LNS usually have either no effect (direct enzyme replacement, fibroblast transplantation, or amnion membrane transplantation) or questionable effects (bone marrow transplantation with wild-type or genetically modified deficient cells) on the CNS involvement (Yatziv *et al.*, 1982; Slavin and Yatziv, 1980; Hoogerbrugge *et*

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*al.*, 1987; Birkenmeier *et al.*, 1991; Birkenmeier, 1991; Sands *et al.*, 1993). This may in part be due to difficulties in delivering enzymes or cells across the blood/brain barrier (BBB). Vascular delivery of purified recombinant β-glucurondidase to neonates does result in detectable levels of activity in the CNS (Vocler *et al.*, 1993), with detectable levels in the meninges, vessels, and ganglia neurons of the peripheral nervous system. Importantly, the enzyme is cleared from the serum within an hour, and intracellular activity diminishes by 4 to 5 days with little to no detectable replacement to CNS neurons. Enzyme replacement to the CNS may therefore be more effectively approached by the direct delivery of transgenes, or transvascular delivery following BBB disruption.

Recently, direct gene replacement to the brain has involved the use of recombinant herpes simplex virus vectors (HSV-I; Palella *et al.*, 1989; Huang *et al.*, 1992; Andersen *et al.*, 1992; Wolfe *et al.*, 1992; Fink *et al.*, 1992), adenoassociated virus (Kaplitt *et al.*, 1994), liposome/plasmids (Davidson and Roessler, 1994; Jiao *et al.*, 1992; Ono *et al.*, 1990), and adenoviruses (Davidson *et al.*, 1993; Bajocchi *et al.*, 1993; Akli *et al.*, 1993; Le Gal La Salle *et al.*, 1993; Davidson *et al.*, 1994a). This chapter describes the results of a series of experiments that demonstrate the effectiveness of recombinant adenoviruses to mediate gene transfer to the CNS using either direct or global delivery methods.

## A. Metabolic Correction of HPRT Deficiency *in Vitro*

Recent publications have focused on the ability of recombinant adenoviruses containing the reporter gene for *Escherichia coli* β-galactosidase to efficiently transduce both neuronal and glial cells in culture or following direct *in vivo* application to striatum, neocortex, ventricles, and the eye (Davidson *et al.*, 1993; Akli *et al.*, 1993; Le Gal La Salle *et al.*, 1993; Bajocchi *et al.*, 1993; Li *et al.*, 1994). The description of these model systems has led to the development of recombinant adenoviruses containing therapeutically relevant transgenes, such as the HPRT gene, with experiments designed to test their efficacy *in vitro* prior to *in vivo* applications.

*In vitro* analysis of recombinant adenovirus containing the rat cDNA was done to determine if HPRT activity could be measured in $HPRT^-$ rat neuroblastoma cells and if the enzyme could salvage radiolabeled purine ([$^3$H]hypoxanthine) substrate to IMP and its catabolites (Davidson *et al.*, 1994). $HPRT^-$ B103-4C cells were grown to near confluence and infected with Ad. RSV*rHPRT* at multiplicities of infection (pfu/cell) ranging from $10^2$ to $10^4$. AdRSV*rHPRT* contains the rat HPRT cDNA flanked by the Rous sarcoma virus (RSV) LTR for promoter activity and a polyadenylation signal from SV40. Infection was done in serum-free media for 4 hr to overnight, and cells were incubated in complete media until time of harvest for enzyme assay or

Southern analysis. Cell passage was required prior to harvest for later time points. Southern blotting detected transgene DNA out to 8 days, with the copy number per cell slowly diminishing from approximately 5 to 0.5 (Fig. 1). Endonuclease restriction using an enzyme which cuts once within the rHPRT sequence revealed that the majority of the transgene persisted as episomal (single fragment of 2.2 kb) DNA.

Enzyme activity persisted out to 28 days, but was highest at Day 15 (Table 1). The fact that HPRT is a relatively stable protein may account for the temporal discrepancy between detectable levels of DNA (by Southern) and detectable enzyme activity (by assay).

Purine salvage was demonstrated following infection of subconfluent B103-4C cells. Cells were infected with Ad.RSV*rHPRT* at a multiplicity of infection (m.o.i.) of $10^3$ for 4 hr in serum-free media, and serum was added to a final concentration of 2% overnight. Cells were then washed with PBS and incubated in complete media for 40 hr. Following a PBS wash, cells were incubated in fresh media containing [$^3$H]hypoxanthine for 2 hr. Cells were harvested and nucleotides extracted and quantified using HPLC (Sidi and Mitchell, 1985). As seen in Table 2, cells transduced with Ad.RSV*rHPRT* were able to salvage [$^3$H]hypoxanthine in contrast to uninfected or mock-infected (Ad.RSV*lacZ*) controls. Radiolabeled pool levels increased in a dose-dependent fashion; [$^3$H]ATP pools at a m.o.i. of $10^4$ were greater than 50-fold higher

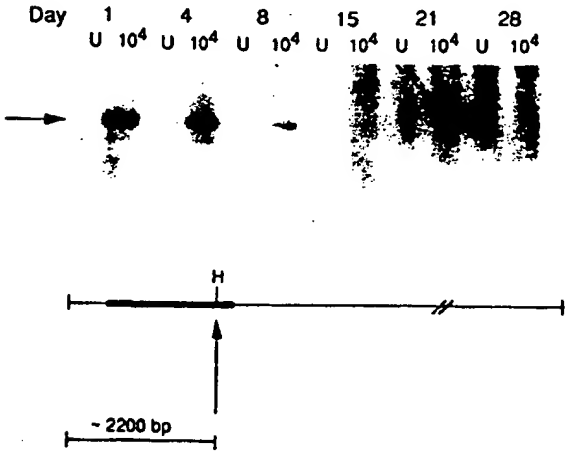


**Figure 1** Southern analysis of DNA isolated from B103-4C cells following Ad.RSV*rHPRT* infection. Cells were infected at a m.o.i. of $10^4$ and harvested at the times indicated. DNA was isolated and digested with *Hpa*I. This enzyme cuts once within the rHPRT sequence in the left half of the full-length recombinant adenoviral genome. Following fractionation on a 0.8% agarose gel, DNA was transferred to nitrocellulose and blots were hybridized to radiolabeled probes specific for rat HPRT. U, uninfected; H, *Hpa*I. The arrow denotes the 2.2-kb fragment predicted following *Hpa*I digestion.

**Table 1**
**HPRT Enzyme Activity in Ad.RSVrHPRT-Infected B103-4C Cells**

| | Multiplicity of infection | | | |
|---|---|---|---|---|
| Day | Un[a] | $10^2$ | $10^3$ | $10^4$ |
| 1 | 1.1[b] | 1.0 | 1.1 | 7.4 |
| 5 | 1.2 | 1.6 | 3.4 | 12.2 |
| 8 | 1.8 | 2.8 | 17.1 | 102.0 |
| 15 | 1.4 | 14.2 | 46.2 | 104.8 |

[a] Uninfected.
[b] CPM/mg total protein $\times 10^{-3}$.

than those seen at a m.o.i. of 1 (Table 2). This data, in conjunction with the Southern and enzyme activity data, indicate that the enzyme is functionally active *in vitro*.

## B. Metabolic Correction of HPRT Deficiency *in Vivo*

Short-term studies designed to examine the ability of Ad.RSV*rHPRT* to modulate purine pool levels or to salvage radiolabeled purines *in vivo* were done in mice deficient in HPRT (Davidson *et al.*, 1994). Direct inoculations into the right striatum were done using 5 $\mu$l of purified adenovirus (experimental or control) at a titer of $1 \times 10^{10}$ pfu/ml. Five days postinoculation, animals were sacrificed, and brain sections surrounding the injection site analyzed for nucleotide pool levels. As shown in Table 3, there was evidence of increased pool levels in the right, infected hemisphere compared to the contralateral controls.

**Table 2**
**[$^3$H]-Nucleotide Pool Levels Following Transduction of B103-4C Neuroblastoma Cells**

| Sample | AMP[a] | ADP | ATP | GDP | GTP |
|---|---|---|---|---|---|
| Uninfected | — | | 11.0 | | |
| Ad.RSV*lacZ*($10^3$)[b] | — | | 7.0 | | |
| Ad.RSV*rHPRT*($10^2$) | — | 182.3 | 105.2 | 644.9 | 90.0 |
| Ad.RSV*rHPRT*($10^3$) | 600.8 | 1140.8 | 822.1 | 10420 | 328.5 |
| Ad.RSV*rHPRT*($10^4$) | 2232.8 | 9900.2 | 5066.5 | 7252 | 9334.6 |

[a] Counts per minute/nmole.
[b] Multiplicity of infection.

**Table 3**
**Nucleotide Pool Levels in Mouse Brain Extracts[a]**

| Hemisphere | AMP | GMP | ADP | GDP | ATP | GTP |
|---|---|---|---|---|---|---|
| Left | 7.16 | 0.54 | 2.28 | 1.16 | 4.32 | 1.01 |
| Right | 8.53 | 0.59 | 3.89 | 1.49 | 5.46 | 1.35 |

[a] nmol/100 μl.

Transgene expression was also assayed *ex vivo* using an *in situ* tissue assay. Sections (2 mm) were overlaid with an enzyme assay mix containing [$^{14}$C]hypoxanthine (100 m*M*, 1 mCi/mmol), 6 m*M* $MgCl_2$, and 10 m*M* PRPP, and incubated for 1 hr at 37°C in a humidified atmosphere. Following incubation, sections were homogenized and nucleotides extracted for analysis of [$^{14}$C]hypoxanthine metabolites. Increased uptake of radiolabeled [$^{14}$C]hypoxanthine was noted in the experimental (Ad.RSV*rHPRT*) vs the contralateral control left hemisphere (Table 4). Animals injected with Ad.RSV*lacZ* did not demonstrate any increases in radiolabeled purine pools compared to the contralateral hemisphere (data not shown), indicating that increased salvage is due to increased expression of transgenic HPRT.

## C. Safety Issues in Nonhuman Primates

We have performed a series of experiments to examine the clinical safety following injection of recombinant adenovirus containing a xenogeneic transgene, the gene for rat HPRT, into primate brain (Doran *et al.*, 1994a). Four rhesus macaques (*Mucaca mulatta*) weighing approximately 3 kg each were injected with adenovirus and followed clinically until the time of sacrifice. Three of the four animals were examined by positron emission tomography (PET), and two of these three by $T_1$ and $T_2$ weighted magnetic resonance imaging (MRI).

*Mucaca mulatta* 1 (MM1) was injected with 200 μl of Ad.RSV*rHPRT* into the left caudate nucleus ($1.6 \times 10^{11}$ pfu/ml). The opposite hemisphere

**Table 4**
**[$^{14}$C]hx Metabolites in Murine Brain Sections**

| Hemisphere | IMP | GMP | ADP | GDP |
|---|---|---|---|---|
| Left | 954[a] | 1938 | 90 | 111 |
| Right | 11524 | 14556 | 212 | 156 |

[a] cpm/section.

was injected with 200 $\mu$l of Ad.RSV*lacZ* ($1.6 \times 10^{11}$ pfu/ml). The animal experienced no perioperative morbidity and throughout the 1-week observation period showed no clinical signs of cerebritis, meningitis, or encephalitis. The animal groomed and fed normally. One week postsurgery, the animal was anesthetized, euthenized, and the brain was perfused with 120 cc of ice-cold saline. The brain was removed and the caudate nucleus blocked into four equal sections. One section was taken for *in situ* RNA hybridization, one for enzyme activity, and two for histochemistry and immunohistochemistry. Enzyme assay and *in situ* RNA analyses were positive for transgene expression (Davidson *et al.*, 1994a). Although clinical signs of disease were not evident, routine H&E revealed a cellular infiltrate consisting of foamy macrophages and lymphocytes. The infiltrate radiated approximately 3 or 4 mm from the injection site, with the number of inflammatory cells diminishing as distance from the injection site increased. Postoperative complete blood counts (CBC) were all within normal limits.

A second long-term nonhuman primate study was also done. MM2 was injected with 200 $\mu$l of Ad.RSV*rHPRT* into the right caudate nucleus. Clinically the animal recovered well, and throughout the 1-year observation period has shown no signs of cerebritis, meningitis, or encephalitis. The animal continues to groom and feed normally. MM2 was studied using the TCC 4600A PET. Cerebral glucose metabolism was studied using bolus injection of 2-[$^{18}$F]fluoro-2-deoxy-D-glucose ([$^{18}$F]FDG), with imaging at 40–60 min after injection. Dopamine receptors in the striatum were studied using infusion of [$^{11}$C]raclopride to a steady state and imaging from 40–60 min. In all studies, there were no significant differences in left vs right hemisphere comparisons of either [$^{18}$F]FDG or [$^{11}$C]raclopride. Comparison of pre- vs postoperative scans were also unremarkable. Serum drawn from the animal 1.5 months postadministration of virus was analyzed for the presence of a cytotoxic T-cell response against Ad.RSV*rHPRT*-infected syngeneic fibroblasts and was negative. Postoperative CBCs have remained within normal limits.

Two animals, MM3 and MM4, were followed by both PET and MRI to clinically evaluate the host response to Ad infection in the CNS. MM3 and MM4 animals were injected with 200 $\mu$l of Ad.RSV*rHPRT* into the right caudate nucleus [total particles (pt) of $10^9$ and $10^{10}$]. The animals recovered well clinically, and throughout the nearly 2-month observation period have shown no signs of cerebritis, meningitis, or encephalitis. Serial MRI and PET scans were initiated 1 week postoperative [$^{18}$F]FDG and [$^{11}$C]raclopride scanning revealed no gross metabolic differences between the injected and uninjected hemisphere. Preoperative MR $T_1$ and $T_2$ imaging (GE Signa, 1.5T; transmit–receive Knee Coil) were normal. $T_1$ (500/18/4; TR/TE/nex) and $T_2$-weighted (2800/92/4) images were obtained with 3-mm slice thickness and 0.5-mm skip. Matrix was $256 \times 256$. MRI scans at 1 week postoperative in MM3, the animal receiving a dose of $10^{10}$ pt, demonstrated only minimal increased signal

intensity on $T_2$ images and minimal enhancement on $T_1$ images along the needle tract. At 4 weeks postoperative there was only minimal increased signal intensity in white matter adjacent to the injection tract on $T_2$ images with no distinct enhancement or altered signal intensity in brain parenchyma on $T_1$ images. PET remained unremarkable at 4 weeks postoperative. At 8 weeks the region of increased signal intensity on $T_2$ images was even less evident and there was no abnormal enhancement. MM4, which received $10^9$ pt, showed changes on $T_2$ weighted MRI at 1 week which were consistent with a 6 × 4 × 9-mm region of edema at the site of virus administration. $T_1$ weighted MR images demonstrated slightly decreased signal intensity in a 1 × 3 × 3-mm area with no enhancement following gadopentetate dimeglumide administration. At 4 weeks postoperative $T_2$ images demonstrated this region of apparent edema to be smaller (4 × 3 × 9 mm) and less intense. However, $T_1$ images without contrast showed increased signal intensity in the same distribution as the $T_2$ signal changes seen at 1 week. With gadopentetate dimeglumide administration there was minimal enhancement in the same region. At 8 weeks the region of increased signal intensity on $T_2$ images was distinctively smaller and there was no abnormal enhancement. The animal remains clinically well. There was minimal enhancement of the meninges over the frontal regions in both postoperative studies in both animals. Serial CBCs have remained within normal limits for both MM3 and MM4 during the experimental period.

These experiments indicate that although the direct administration of recombinant adenovirus to brain induces a notable immune response, the response resolves over time. The consequence of redosing in the same animal, which is an important issue as no current generation of viruses can direct transgene expression indefinitely, remains to be determined.

### D. Alternate Delivery Strategies

A major obstacle to the delivery of recombinant viruses to the CNS is the presence of a BBB formed by the brain capillary endothelial cells and a blood–CSF barrier formed by the choroid plexus epithelial cells and the meninges (Rapoport and Robinson, 1986). These barriers greatly impede the passage of most polar molecules from the blood to the brain. Although the interface between the CSF and brain is lined by ependymal cells in the cerebral ventricles and by pial cells in the subarachnoid space, these cells do not form barriers. Several methods have been developed to reversibly and transiently open the BBB including intracarotid infusion of hyperosmotic solutions (Neuwelt *et al.*, 1979), polycations (Hardebo and Kahrstrom, 1985; Strausbaugh, 1987; Westergren and Johansson, 1993), oleic acid (Sztriha and Betz, 1991), and histamine (Gross *et al.*, 1982).

Osmotic BBB disruption requires intracarotid infusion of extremely hypertonic solutions (e.g., 1.6 *M* arabinose) for 20 sec or more. The barrier

remains open for up to 2 hr (Rapoport *et al.*, 1980). The mechanism of barrier opening appears to involve separation of tight junctions (Dorovini-Zis *et al.*, 1984) perhaps due to shrinkage of the endothelial cells (Dorovini-Zis *et al.*, 1983). This technique has been successfully adapted for the delivery of chemotherapeutic agents in humans (Neuwelt *et al.*, 1986). A possible limitation to this approach for delivery of viruses is that the barrier may not open enough to accommodate the size of adenovirus (Ad5 has a diameter of about 70 nm). However, Neuwelt *et al.* (1991) showed increased uptake of ultraviolet-inactivated HSV-1 (native size of 120 nm in size) following osmotic disruption of the BBB and intravascular administration of virus. Thus, preliminary experiments were done to determine if recombinant adenovirus can cross the BBB (Doran *et al.*, 1994b).

Male Sprague–Dawley rats weighing 250 gm were anesthesized with isoflourane (5% induction, 2% maintenance) and the right carotid artery was exposed through a ventral neck incision. A catheter filled with heparinized saline was introduced into the right external carotid artery and advanced retrograde to the bifurcation of the common carotid artery. Evans blue solution (2 ml/kg of 2 g/100 ml solution) was given intravenously 5 min prior to blood–brain barrier disruption to serve as a marker of altered BBB integrity. Blood–brain barrier disruption was then performed as previously described (Neuwelt *et al.*, 1991). In six animals, mannitol (25%, 37°C) was infused cephalad into the internal carotid artery at a rate of 0.12 ml/sec for 30 sec. Control animals were infused with 0.9% saline, 37°C, into the internal carotid artery at the same rate and duration. After waiting 60 sec, $5 \times 10^{11}$ particles of Ad.RSV*lacZ* in 1 ml of 0.9% saline were infused in all animals over a period of 60 sec. Animals were then recovered and housed in a biocontainment facility.

The degree of blood–brain barrier disruption based on Evans blue staining was graded from 0 to 3+, as previously described (Rapoport *et al.*, 1980). The Evans blue–albumin complex does not cross the normal BBB and the degree of staining in the osmotically disrupted brain is a measure of BBB opening. Of the six rats receiving intracarotid mannitol, three had 2+ Evans blue staining and three had 1+ staining of the ipsilateral cerebral hemisphere (Fig. 2). As these animals were sacrificed 4 days after barrier disruption, and as Evans blue is gradually cleared from the disrupted brain, it is likely that the degree of barrier disruption was greater at the actual time of adenoviral infusion. No Evans blue staining was found in brains of animals receiving intracarotid saline.

Following X-gal staining, brain sections from rats that had undergone mannitol-induced BBB disruption followed by intracarotid Ad.RSV*lacZ* injection were all *E. coli lacZ* positive in pericapillary astrocyte. (Fig. 2). No intracerebral *E. coli lacZ* activity was found in control animals receiving intracarotid saline followed by Ad.RSV*lacZ*. In animals receiving intracarotid mannitol, X-gal-stained cells were found almost exclusively in the disrupted right hemi-

sphere. Rare positive cells were found in the extreme medial aspect of the opposite hemisphere, in the so-called "watershed" areas of vascular anastamoses. The highest concentration of X-gal-stained cells was found in the cerebral cortex, followed by the deep gray nuclei. The number of positive cells correlated with the degree of Evans blue staining: hemispheres with 2+ Evans blue staining typically had 5–10 *lac*Z-positive cortical cells per low-powered field, while hemispheres with 1+ Evans blue staining had 0–2 cortical cells per low-powered field.

*Escherichia coli lac*Z activity was found only in astrocytes as shown morphologically by X-gal staining. X-gal staining was not seen in neurons or ependymal cells. X-gal-positive astrocytes were found primarily adjacent to capillaries (Fig. 2). TEM and immunohistochemistry were also done to confirm the identity of the transduced cells of the cerebral cortex based on ultrastructural criteria (data not shown). Histochemistry, immunohistochemistry, and TEM failed to detect transduced neurons. The most likely explanation for this finding is the normal anatomic relationship between cerebral capillaries and astrocytes. Surrounding the basement membranes of the capillary endothelium are the foot processes of astrocytes. After intracarotid infusion of mannitol, the capillary endothelial cells presumably shrink and the tight junctions are temporarily opened, allowing the recombinant adenovirus to leave the cerebral vasculature and enter the perivascular space. The first structure then encountered would be the foot processes of astrocytes. The capacity of astrocyte transduction following BBB -disruption to globally correct the neuropathology in animal models of metabolic disease is currently under study.

## II. Application to the Treatment of CNS Neoplasms

In addition to the application for the treatment of inherited metabolic disorders, recombinant adenoviruses may be useful in the treatment of CNS neoplasms. Over 17,000 new cases of primary intracranial tumors are diagnosed annually. The incidence of all primary brain tumors in the United States increases with increasing age, extending from 2.3 per 100,000 during childhood to a peak rate of 20.4 per 100,000 in the 55- to 75-year-old age group. Gliomas are the most frequently encountered brain tumor in all age groups, accounting for approximately 57% of all primary intracranial brain neoplasms (New, 1993; Levin *et al.*, 1993). They encompass a range of tumors including astrocytomas, glioblastoma, oligodendrogliomas, and ependymal tumors. These tumors are thought to arise from the neoplastic transformation of differentiated cells at loci of glial cell development. Gliomas are usually nonencapsulated and infiltrate into the surrounding brain parenchyma.

Because of the extremely poor prognosis, alternate management strategies are continually being developed. Gene therapy has recently been investigated as an alternative for brain tumor treatment. Viral or synthetic transducing agents containing a "suicide gene," usually thymidine kinase (TK) from HSV-I (Borrelli *et al.*, 1988; Barba *et al.*, 1993; Moolten, 1986), are used to deliver the gene to the tumor (Moolten and Wells, 1990; Ezzeddine *et al.*, 1991; Culver *et al.*, 1992; Ram *et al.*, 1993; Plautz *et al.*, 1991). The expressed gene is not in itself toxic to the cell. However, administration of an antiviral 2'-deoxyguanosine analog drug, ganciclovir (Cytovene; GCV), results in the formation of toxic phosphorylated guanosine intermediates in cells expressing the transduced TK (Shewach *et al.*, 1994). Cell death occurs only in actively dividing cells, presumably as a result of the incorporation of the toxic nucleoside intermediates into DNA. Because the cell must be actively dividing to respond to the harmful effects of the drug, the *tk* gene is a good candidate for gene therapy treatment of gliomas (Moolten and Wells, 1990; Ezzeddine *et al.*, 1991; Culver *et al.*, 1992; Ram *et al.*, 1993). Importantly, surrounding glial and neuronal tissue have an extremely slow mitotic rate imparting a protective effect from the phosphorylated analog.

A variety of transducing agents has been used to transfer DNA to the tumor mass, including liposomes (Yoshida *et al.*, 1992) and retroviral vectors (Chu *et al.*, 1993; Culver *et al.*, 1992; Oldfield *et al.*, 1993; Ram *et al.*, 1993, 1992; Short *et al.*, 1990; Takamiya *et al.*, 1992, 1993). Gene transfer with retroviral vectors is restricted to actively dividing cells, which is an attractive feature when targeting malignant cells amidst a background of quiescent, nonproliferating cells. A major drawback, however, is that low efficiency of gene transfer and inability to obtain high titers necessitates the *in situ* introduction of producer cell lines to obtain optimal levels of gene transfer. Also, the introduction of mouse fibroblast (producer cells) will likely confound readministration of producer cells due to host immune response. In contrast, recombinant adenoviruses possess a number of characteristics which make them attractive candidates for gene therapy (Kozarsky and Wilson, 1993). Adenoviruses can be grown to very high titers, $10^{13}$ plaque-forming units (pfu) vs $10^{5-7}$ pfu for retroviruses, and have no requirement for cell division for transgene expression (Mulligan, 1993). This is an important feature as only approximately 10% of the tumor is actively dividing at any one time, limiting the level of transduction that can occur with retroviral producer cells.

Ad.RSV*tk*, a recombinant adenovirus designed to deliver *tk* from HSV-I, has been shown to infect brain tumor cells *in vitro* and *in vivo* and render them susceptible to ganciclovir toxicity (Shewach *et al.*, 1994; Smythe *et al.*, 1994, 1995; Ram, 1994; Chen *et al.*, 1994; Davidson *et al.*, 1994b). When the virus is injected stereotactically into the tumor, the risk of infecting nontumor tissue is minimal. Also, Ad.RSV*tk*/GCV therapy in nontumor-bearing tissue in rodents does not appear to induce damage; animals are clinically

normal and there is no gross evidence of disease. Similar studies are currently being carried out in nonhuman primates.

### A. Transduction of Rodent Tumor Cell Lines *in Vitro*

Initial experiments have been done to determine the ability of Ad.RSV*tk* to confer GCV toxicity to glioma cells in culture. TK-deficient C6BU1 cells, which are derived from C6 rat glioma cells, were infected at varying m.o.i.s (pfu/cell) (Shewach *et al.*, 1994; Takamiya *et al.*, 1992). As seen in Fig. 3, a linear relationship exists between m.o.i. and HSV-TK activity over a range of 2 to 10,000 m.o.i. without apparent saturation. Transduction with Ad.RSV*tk* resulted in HSV-TK activity levels that were approximately 600-fold higher than those with retroviral-mediated HSV-TK transfer into C6BU1 cells (Shewach *et al.*, 1994).

The high levels of HSV-TK activity in the C6BU1 cells would be expected to lead to greater sensitivity to the cytotoxic effects of GCV. As shown in Fig. 4, cytotoxicity was enhanced with increasing m.o.i. In addition, the $IC_{50}$ values for the drug decreased more than 20-fold with increasing m.o.i. from 0.077 $\mu M$ at an m.o.i. of 10 to 0.0037 $\mu M$ at an m.o.i. of 1000. A similar dose-dependent decrease in $IC_{50}$ is seen in 9L glioma cells (data not shown). This allows for the use of lower systemic doses of GCV in animals following Ad.RSV*tk* gene transfer. The results of these experiments are described in detail in Shewach *et al.* (1994).

Mixing experiments with Ad.RSV*tk*-infected mesothelioma cells have demonstrated that as little as 10% Ad.RSV*tk*-infected cells are capable of producing a tumoricidal effect (Smythe *et al.*, 1995). These experiments have

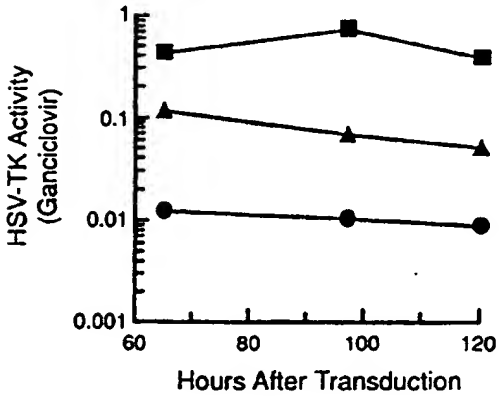


**Figure 3** Relationship between m.o.i. and HSV-TK activity. C6BU1 cells were transduced with Ad.RSV*tk* 3 days prior to harvesting for measurement of HSV-TK activity. HSV-TK activity is expressed as nmol/hr per $10^6$ cells.

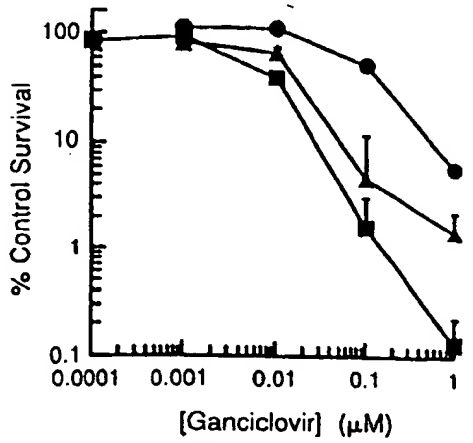


**Figure 4** Effect of increasing m.o.i.s on the cytotoxicity of GCV. Cells were transduced with Ad.RSV*tk* at m.o.i.s of (●) 10, (▲), 100, or (■) 1000. Clonogenic cell survival was determined at each m.o.i. (1).

also been done in rat glioblastoma cell line C6 with similar results (Chen *et al.*, 1994).

## B. *In Vivo* Studies in Rodent Models

### 1. HSV-TK Enzyme Activity and Effects on Growth Kinetics

The ability of Ad.RSV*tk* to confer susceptibility to GCV *in vivo* was demonstrated in an animal model of glioblastoma (Ross *et al.*, 1995). 9L glioma cells were grown as monolayers and $10^5$ cells injected into the right striatum of Fischer 344 rats 3 mm from the cortical surface. Initial studies were done to determine HSV-I TK activity on Days 1–3 following Ad.RSV*tk* injection into tumors. Enzyme assay of glioma tissue removed from animals sacrificed at Day 1 postadministration of virus was 0.514 ± 0.26 nmol phosphorylated GCV/gram tumor tissue ($n = 5$). By Day 2, this level had increased to 0.9851 ± 0.28 ($n = 5$). Levels at Day 3 were similar ($n = 3$). Based on this information, twice daily injections of GCV (15 mg/kg) were initiated 24 to 30 hr postvirus administration in all animal studies.

Growth kinetics were determined by MRI scans that were initiated between 8 and 10 days postimplantation and repeated every other day to obtain volume measurements of tumors. At approximately Day 16, tumors were injected with $2.5 \times 10^8$ pfu of freshly prepared Ad.RSV*tk* in PBS. GCV treatment (15 mg/kg b.i.d. for 10 days) was administered ip with the first injection occurring 24 hr post-Ad.RSV*tk* infection. Note that 15 mg/kg was effective, compared to 150 mg/kg in other studies (Moolten and Wells, 1990; Ezzeddine *et al.*, 1991; Culver *et al.*, 1992; Ram *et al.*, 1993; Plautz *et al.*, 1991).

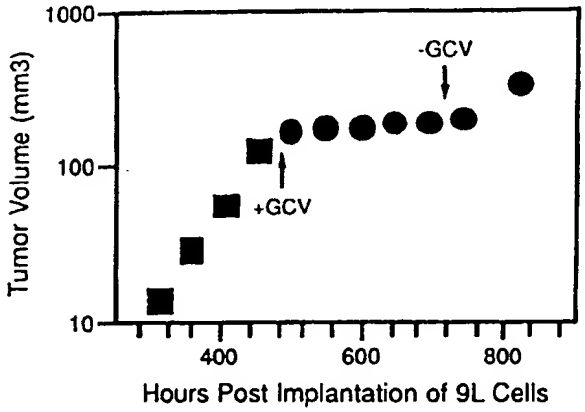


**Figure 5** A representative growth curve for an intracranial 9L glioma treated with Ad.RSV*tk* and GCV. Note the marked growth retardation during the treatment period.

A representative curve demonstrating the growth kinetics of the tumor prior, during, and post-treatment is shown in Fig. 5. Marked growth retardation represented by an increase in tumor doubling time ($T_d$) from 53 ± 2.5 to 403 ± 111 hr (range 102 to 964 hr) during GCV administration was observed in 9 of 11 animals. Controls receiving Ad.RSV*lac*Z/GCV showed no change in $T_d$. Data summarized from these 9 animals are shown in Table 5.

**Table 5**
**Tumor Doubling Time Pre- and Post-Ad.RSV*tk* and GCV Treatment**

| Rat No. | $T_d$ (pre)[a] | $T_d$ (post) |
|---|---|---|
| 1 | 44 | 964 |
| 2 | 59 | 276 |
| 3 | 51 | 206 |
| 4 | 59 | ∞[b] |
| 5 | 43 | 213 |
| 6 | 37 | 140 |
| 7 | 65 | 102 |
| 8 | 56 | 192 |
| 9 | 55 | 183 |
| Mean ± SE | 53 ± 2.5 | 403 ± 111 |

[a] $T_d$ (tumor doubling time) is given in hours.
[b] Tumor volume decreased. $T_d$ of 1000 hours used for statistical analysis.

2. Survival Study

Standard survival studies were done on rats implanted with 9L glioma cells (Ross *et al.*, 1995). Two cohorts of animals consisting of eight animals each were implanted with 9L cells. A third cohort of eight animals remained tumor free. All animals were inoculated on Day 7 with 20 μl of Ad.RSV*tk* (eight tumor-bearing and eight nontumor bearing animals; $1.2 \times 10^{11}$ pfu/ml, $4.6 \times 10^{12}$ pt/ml) or 20 μl of Ad.RSV*lacZ* (tumor bearing; $1.6 \times 10^{11}$ pfu/ml, $5.5 \times 10^{12}$ pt/ml). GCV was given twice daily, 5 mg/kg, with the first dose given 24 hr following Ad infection. GCV treatment was continued for 14 days. Figure 6 demonstrates that (i) animals receiving Ad.RSV*tk*/GCV survived significantly longer than those receiving control virus (Ad.RSV*lacZ*/GCV), and (ii) nontumor bearing animals receiving Ad.RSV*tk*/GCV therapy were clinically normal and did not succumb to any untoward side effects of infecting normal brain tissue with the HSVtk gene, and subsequent conversion of GCV to the toxic triphosphate form. To summarize, rats receiving Ad.RSV*tk* and GCV treatment ($n = 8$) survived significantly longer (50% increase in survival time) than animals receiving control adenovirus (Ad.RSV*lacZ*; $n = 8$). Mean survival ± SEM was 2.5 ± 1.15 days in Ad.RSV*lacZ*/GCV rats compared to 38 ± 4.49 days in Ad.RSV*tk*/GCV-treated animals. The effect of Ad.RSV*tk*/GCV on normal brain was unremarkable ($n = 8$).

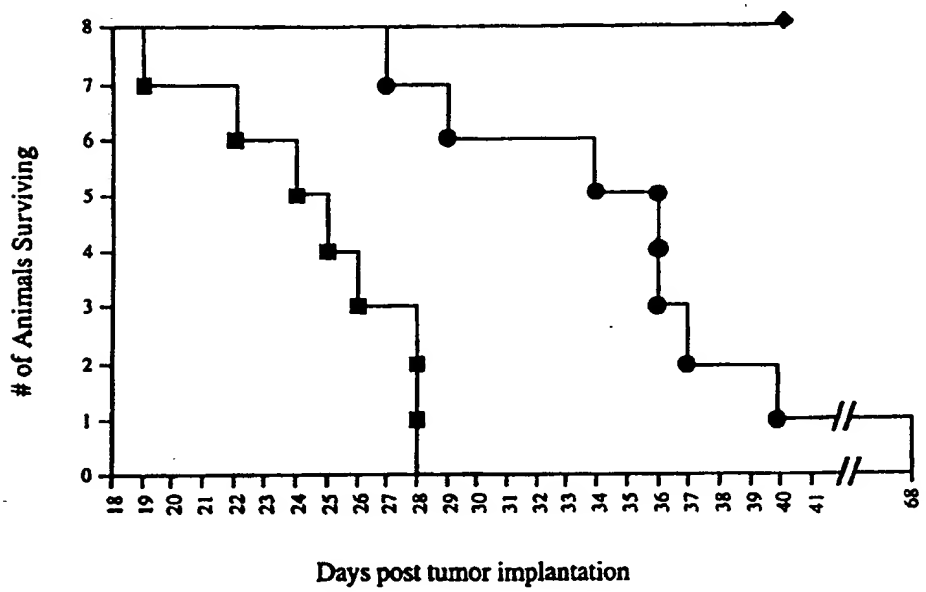


**Figure 6** Survival study of rats following Ad.RSV*lacZ* and Ad.RSV*tk* injections. See text for a description of the experiments. ■, Ad.RSV*lacZ*/GCV; ●, Ad.RSV*tk*/GCV; ◆, Ad.RSV*tk*/GCV (no tumor).

3. *In Vivo* Spectroscopy Measurements

MRS can be used to study tumor metabolism *in vivo* and, as such, is a powerful tool to assess treatment efficacies (Ross *et al.*, 1992). The metabolic parameters which can be analyzed using MRS differ substantially and are therefore complementary to those provided by PET. Thus, MRS offers us an additional tool to determine the *in vivo* effects of the therapy. In addition to the detection of lipid and lactate, which are indicators of necrosis, MRS can localize neuronal-specific metabolites (e.g., *N*-acetyl aspartate). Because spectroscopic changes are usually observed from hours to a day posttherapy, rather than days to weeks with MRI, this technique should provide evidence for therapeutic efficacy which will precede morphologic changes such as decreased tumor volume. This data could also yield valuable insights into the duration of therapeutic effects which would assist in the optimization of redosing strategies.

A preliminary study was designed using MRS to determine if spectroscopic changes occurred following Ad.RSV*tk*/GCV treatment prior to notable radiographic changes. In these studies, spatially localized $^1$H MRS studies were accomplished on Ad.RSV*tk*/GCV-treated gliomas during the time course of the treatment protocol. Localized *in vivo* $^1$H spectra were acquired from 20-$\mu$l tissue volumes using adiabatic pulses combined with one-dimensional spectroscopic imaging along a column selected by two-dimensional ISIS (Ross *et al.*, 1992). Metabolites which are notably different between glioma tissue and normal brain are diminished concentrations of *N*-acetyl-aspartate and creatine and increased levels of choline (Ross *et al.*, 1995). A representative spectra from a tumor-bearing and AdRSV*tk*/GCV-treated animal is shown in Fig. 7.

## III. Future Directions—Vector Development

There are several requirements which must be met before replication-deficient adenovirus can be considered a viable vehicle for the delivery of genes into the brain parenchyma for correction of inherited disorders of metabolism. In addition to its ability to infect and express in multiple cell types relatively efficiently, transgene expression must persist for long periods of time and the adenovirus must be amenable to redosing when transgene expression diminishes. The most significant problem associated with persistence of transgene expression may be directly related to the immunotoxicity of the virus. Akli and others (Akli *et al.*, 1993; Bajocchi *et al.*, 1993; Davidson *et al.*, 1993) demonstrated an acute toxicity following direct injection into brain, particularly at high viral titers. This acute response also occurs in lungs and is unrelated

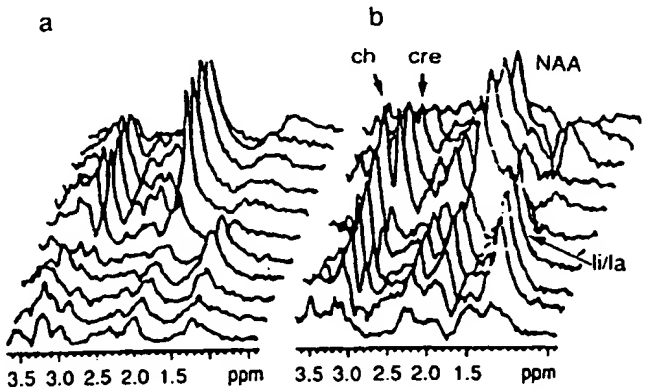


**Figure 7** Each figure displays a series of spectra from 25-μl voxels along a two-dimensional column of the rat. Spectra of untreated gliomas (a) is distinctive, with an absence of *N*-acetyl aspartate and reduced levels of creatine in the right hemisphere (tumor bearing; first five spectra in both panels from front to back) compared to the contralateral (nontumor-bearing; last six spectra) hemisphere. The lipid/lactate resonance from untreated gliomas and healthy brain is barely visible. In the GCV–Ad.RSV*tk*-treated glioma (b), $^1$H spectra revealed an increase in the lipid/lactate resonance and a decrease in creatine levels. li, lipid; la, lactate; NAA, *N*-acetyl aspartate; ch, choline; cre, creatine.

to viral or transgene expression (McCoy *et al.*, 1994). At all titers, transgene expression diminishes over time. This is due in part to a CD8-dependent host response which can be tempered following backbone modifications to further cripple expression of immunogenic viral proteins (Engelhardt *et al.*, 1994a,b; Yang *et al.*, 1994). Whether or not such modifications result in prolonged expression in the CNS as a result of diminished immune response remains to be determined.

Vector improvements which result in persistence are less an obstacle for the use of adenovirus in anticancer therapies. However, the efficient delivery of suicide genes to effect a complete tumoridical response will no doubt require redosing of the virus. Whether or not vector modifications can be made to overcome the obstacles inherent in the current generation of adenoviral vectors is an area of intense research interest.

In summary, adenoviral vectors will undoubtedly lead the field in applications directed at the study of CNS biochemistry, physiology, and neuropharmacology because of their relative ease in generation, manipulation, and application (high efficiency of infection with low toxicity) compared to other viral vectors. The direct application to CNS disease in humans, however, awaits further development and thorough safety and efficacy studies in animal models of disease.

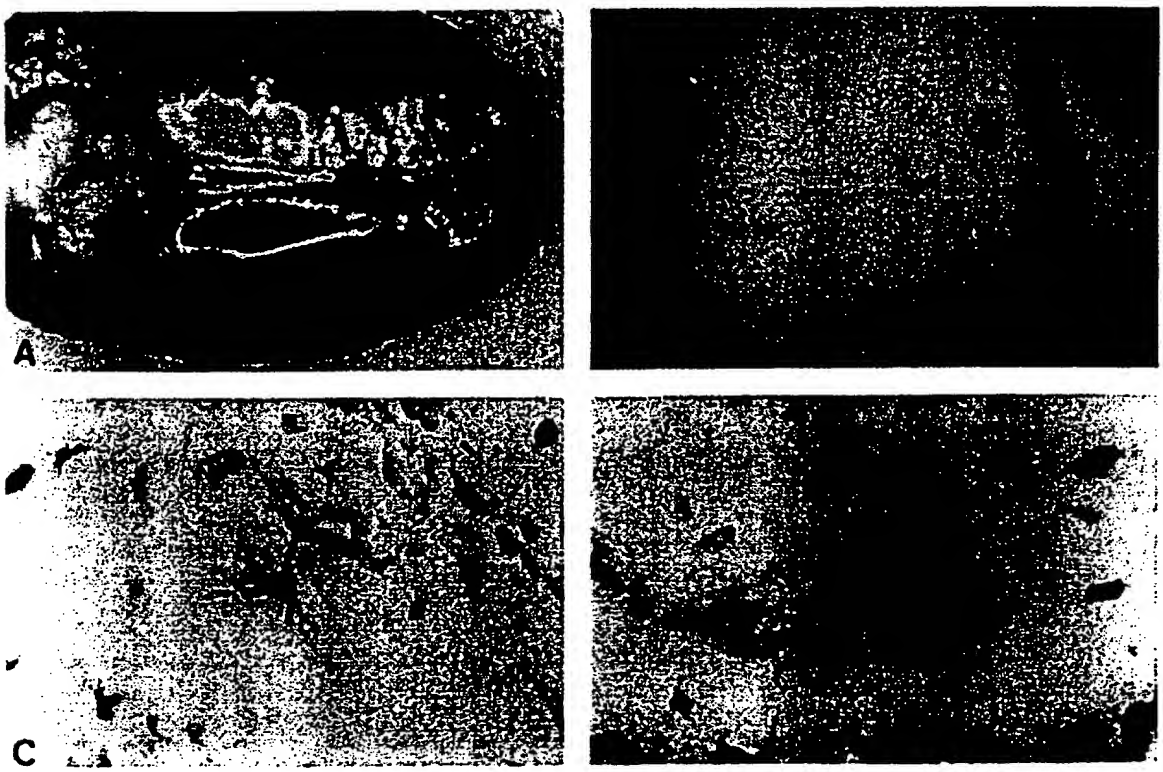


**Figure 2** (A) Evans blue staining (graded 2+) of the right cerebral hemisphere following osmotic disruption with intracarotid mannitol. (B) Low-powered view (100×) of a X-gal-stained rat cerebral cortex following BBB disruption and intracarotid infusion of Ad.RSV*lacZ*. Note the blue-stained cells scattered throughout the cerebral cortex, indicating transgene expression. (C and D) High-powered view (400×) of astrocytes from the cerebral cortex expressing transgenic *lacZ*. In D, the foot process of the astrocyte is in direct contact with an adjacent capillary.

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